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(54) Parathyroid hormone derivatives.

(57) Disclosed are peptides and salts thereof represented by general formul

R<sub>1</sub>-Val-Ser-Glu-Ile-Gln-Leu-R<sub>2</sub>-His-Asn-R<sub>3</sub>-R<sub>4</sub>-R<sub>5</sub>-  
His-Leu-Asn-Ser-R<sub>6</sub>-R<sub>7</sub>-Arg-R<sub>8</sub>-Glu-R<sub>9</sub>-Leu-R<sub>10</sub>-R<sub>11</sub>-R<sub>12</sub>-  
Leu-Gln-Asp-Val-His-Asn-R<sub>13</sub>

wherein R<sub>1</sub> represents Ser or Aib; R<sub>2</sub> represents Met or a naturally occurring hydrophobic amino acid; R<sub>3</sub> represents Leu, Ser, Lys or an aromatic amino acid; R<sub>4</sub> represents Gly or a D- $\alpha$ -amino acid; R<sub>5</sub> represents Lys or Leu; R<sub>6</sub> represents Met or a naturally occurring hydrophobic amino acid; R<sub>7</sub> represents Glu or a basic amino acid; R<sub>8</sub> represents Val or basic amino acid; R<sub>9</sub> represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R<sub>10</sub> represents Arg or His; R<sub>11</sub> represents Lys or His; R<sub>12</sub> represents Lys, Gln or Leu; and R<sub>13</sub> represents Phe or Phe-NH<sub>2</sub>; except that simultaneously R<sub>1</sub> consists of Ser, R<sub>2</sub> consists of Met, R<sub>3</sub> consists of Leu, R<sub>4</sub> consists of Gly, D-Ala or D-Pro, R<sub>5</sub> consists of Lys, R<sub>6</sub> consists of Met, R<sub>7</sub> consists of Glu, R<sub>8</sub> consists of Val, R<sub>9</sub> consists of Trp, R<sub>10</sub> consists of Arg, R<sub>11</sub> consists of Lys and R<sub>12</sub> consists of Lys.

The parathyroid hormone (1-34) analogues are useful in hormone therapy.

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BACKGROUND OF THE INVENTION

The present invention relates to novel parathyroid hormone peptide derivatives useful in hormone therapy.

5 Parathyroid hormone (PTH) is synthesized in the parathyroid, and plays an important role in controlling blood calcium concentrations or phosphoric acid ion concentrations by acting on the bone, the kidney and the intestine which are its target organs. PTH is a peptide hormone consisting of 84 amino acids, and the biological action thereof can be reproduced by a peptide fragment of an N-terminal (1 through 34 amino acid) portion, G. W. Tregebar et al., *Endocrinology* 93, 1349-1353 (1973).

10 The amino acid sequence of the peptide fragment of the N-terminal (1 through 34 amino acid) portion of this human type PTH (this peptide fragment is hereinafter abbreviated as human PTH(1-34) or hPTH(1-34)) is as follows:

15            1    2    3    4    5    6    7    8    9    10   11   12   13  
H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-

20            14   15   16   17   18   19   20   21   22   23   24   25   26  
His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-

25            27   28   29   30   31   32   33   34  
Lys-Leu-Gln-Asp-Val-His-Asn-Phe-OH (SEQ ID NO:1)

From the biological action of PTH, it is expected that the use of PTH as a drug will provide a drug 25 useful for various bone diseases and the like. However, the following properties of the peptide make it difficult:

- (1) The peptide is easily decomposed by various enzymes within the body;
- (2) The absorption efficiency of the peptide into the body through various routes is very low; and
- (3) The peptide is unstable to various physicochemical conditions such as oxidation.

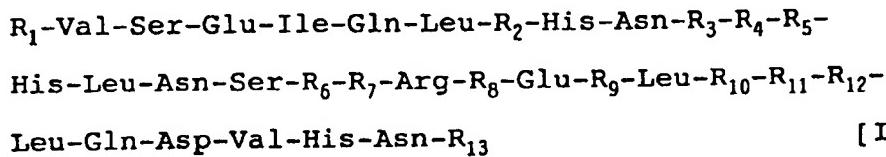
In order to solve such problems and to understand the relationship between structure and activity of the above hormone, various derivatives have been synthesized for the PTH(1-34) fragment. While, such 30 syntheses have been conducted for bovine PTH(1-34), few examples are known for the human PTH(1-34). For example in one such derivative, when the C-terminus Phe of the human PTH(1-34) is converted to Phe-NH<sub>2</sub>, an increase in activity is observed (Japanese Patent Unexamined Publication No. 58-96052). This 35 increase in activity is believed to be due to inhibition of carboxypeptidase which decomposes the hormone. Further, human PTH(1-34) contains two Met residues. A molecule in which these Met residues are substituted with Nle residues prevents the hormone from losing its activity due to oxidation (Japanese Patent Unexamined Publication No. 61-24598).

40 SUMMARY OF THE INVENTION

In order to solve the above described problems, the inventors substituted one or more amino acid residue of human PTH(1-34) by chemical synthesis. The substitutions effect the resulting molecule's resistance to various proteases, its two-dimensional structure as well as its reaction in 45 hydrophilic/hydrophobic or ionic media. By substituting the amino acid unstable to acidic, basic or oxidation conditions with an amino acid stable to these conditions, a molecule which is an object of the present invention is synthesized. In addition, clinically effective PTH analogues have been synthesized in accordance with the present invention.

Namely, the present invention provides:

50 (1) a peptide represented by general formula [I] or a salt thereof:



[I]

wherein R<sub>1</sub> represents Ser or Aib; R<sub>2</sub> represents Met or naturally occurring hydrophobic amino acid; R<sub>3</sub> represents Leu, Ser, Lys or an aromatic amino acid; R<sub>4</sub> represents Gly or a D- $\alpha$ -amino acid; R<sub>5</sub> represents Lys or Leu; R<sub>6</sub> represents Met or naturally occurring hydrophobic amino acid; R<sub>7</sub> represents Glu or a basic amino acid; R<sub>8</sub> represents Val or a basic amino acid; R<sub>9</sub> represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R<sub>10</sub> represents Arg or His; R<sub>11</sub> represents Lys or His; R<sub>12</sub> represents Lys, Gln or Leu; and R<sub>13</sub> represents Phe or Phe-NH<sub>2</sub>. However, except that simultaneously R<sub>1</sub> consists of Ser, R<sub>2</sub> consists of Met, R<sub>3</sub> consists of Leu, R<sub>4</sub> consists of Gly, D-Ala or D-Pro, R<sub>5</sub> consists of Lys, R<sub>6</sub> consists of Met, R<sub>7</sub> consists of Glu, R<sub>8</sub> consists of Val, R<sub>9</sub> consists of Trp, R<sub>10</sub> consists of Arg, R<sub>11</sub> consists of Lys and R<sub>12</sub> consists of Lys (SEQ ID NO:2).

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#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Naturally occurring hydrophobic amino acids of R<sub>2</sub> and R<sub>6</sub> mean hydrophobic ones among amino acids which consist of natural proteins originating from animal, plant or microorganisms, and including Leu, Ile, Val, Phe and Trp. Aromatic amino acids of R<sub>3</sub> include Phe,  $\beta$ -naphthyl Ala, Trp and Tyr. D- $\alpha$ -amino acids of R<sub>4</sub> may be any D- $\alpha$ -amino acid, and includes D-Leu, D-Ile, D-Nle, D-Val, D-Ser, D-Ser(But), D-Abu, D-Thr, D-Nva, D-Met, D-B-naphthyl-Ala, D-Trp, D-Tyr, D-Lys, D-Lys(Fmoc), D-Phe and D-Asn. Generally, neutral amino acids are preferable including D-Ser, D-Leu, D-naphthyl Ala, D-Trp, D-Asn and D-Tyr. Basic amino acids of R<sub>7</sub> and R<sub>8</sub> include Arg, Lys, Asn and His. The substitution of the above-described groups may be at one or more positions and the substitution combination up to three positions is preferable. Accordingly, a peptide or a salt thereof of the formula [1] may simultaneously have 10 to 12 amino acids optionally selected from a group consisting of Ser for R<sub>1</sub>, Met for R<sub>2</sub>, Leu for R<sub>3</sub>, Gly for R<sub>4</sub>, Lys for R<sub>5</sub>, Met for R<sub>6</sub>, Glu for R<sub>7</sub>, Val for R<sub>8</sub>, Trp for R<sub>9</sub>, Arg for R<sub>10</sub>, Lys for R<sub>11</sub>, Lys for R<sub>12</sub> and Phe for R<sub>13</sub>.

Peptide synthesis in the present invention can be carried out by the use of an automatic peptide synthesizer. The method of R. B. Merrifield *Advances in Enzymology* 32, 221-296 (1969) applies correspondingly to a basic synthesis course. In this method, the amino acid of the carboxyl terminus is covalently bound to a resin carrier, and elimination of a protective group of an  $\alpha$ -amino group and condensation of a protected amino acid are repeated in turn to extend a peptide chain to the amino terminus, thereby obtaining a protected peptide resin having a desired amino acid sequence. This method is based on the above-described principle. The condensation of each amino acid and the elimination of the protective groups of the  $\alpha$ -amino groups are performed under approximately similar conditions, and purification of intermediates is not conducted. In synthesizing peptides, therefore, skill of a high order generally is not required. Moreover, the peptides are rapidly synthesized by this method, so that this method is very convenient to synthesize various peptides. The protected peptide resin thus obtained is reacted with, for example, anhydrous hydrogen fluoride, trifluoromethanesulfonic acid or trifluoroacetic acid in the coexistence of various additives, whereby elimination of the peptide from the resin and removal of all protective groups can be achieved in one step.

The resulting crude peptide can be purified by known means for purifying peptides or proteins. Examples of such means include column chromatography under various principles such as gel filtration, ion exchange chromatography using a cation exchange resin or an anion exchange resin, hydrophobic chromatography and partition adsorption chromatography, and high performance liquid chromatography.

The peptides of the present invention can be obtained in various salt forms. Examples of the salts include salts of inorganic acids, salts of organic acids such as formic acid, acetic acid, tartaric acid and citric acid, salts of inorganic bases such as sodium and ammonium, and salts of organic bases such as triethylamine, ethylamine and methylamine.

The human PTH(1-34) derivative peptides represented by general formula [1] of the present invention can be used as therapeutic agents for osteoporosis, hypoparathyroidism and hypertension. The forms thereof include injections, nasotracheal absorption agents, perrectum absorption agents, transvaginal absorption agents, percutaneous absorption agents and eye drops. In some cases, they are orally administered.

When the peptides are used as such therapeutic agents, effective amounts thereof are used to treat mammals, especially humans. Although they are generally used within the range of 1 ng to 100  $\mu$ g/kg of weight, precise amounts thereof may be determined by those skilled in the art.

When the peptides are used as the therapeutic agents, they must be carefully purified so as to contain no bacteria and no pyrogens.

The peptides, when used as the therapeutic agents for osteoporosis and the like, can be administered parenterally in the form of the above-described injections, nasotracheal absorption agents, perrectum absorption agents, transvaginal absorption agents, percutaneous absorption agents or eye drops, solely or

in combination with pharmaceutically acceptable carriers, excipients or diluents. In the case of the injections, it is appropriate that the peptides are given to adults in a dose of 50 ng/kg to 5 mg/kg for 1 to 3 days, and preferably in a dose of 1 to 500 µg/kg for 1 to 3 days. For the injections, it is appropriate that the concentration of the therapeutic agent is 10 to 100 µg/ml.

5 When nucleotides, amino acids and the like are indicated by abbreviations in this specification, the abbreviations adopted by the IUPAC-IUB Commission on Biochemical Nomenclature or those commonly used in the art are employed. For example, the following abbreviations are used. When the amino acids are capable of existing as optical isomers, it is understood that the L-forms are represented unless otherwise specified.

10	Gly or G :	Glycine
	Ala or A :	Alanine
	Val or V :	Valine
	Leu or L :	Leucine
	Ile or I :	Isoleucine
15	Ser or S :	Serine
	Thr or T :	Threonine
	Cys or C :	Cysteine
	Met or M :	Methionine
	Glu or E :	Glutamic acid
20	Asp or D :	Aspartic acid
	Lys or K :	Lysine
	Arg or R :	Arginine
	His or H :	Histidine
	Phe or F :	Phenylalanine
25	Tyr or Y :	Tyrosine
	Trp or W :	Tryptophan
	Pro or P :	Proline
	Asn or N :	Asparagine
	Gln or Q :	Glutamine
30	Aib :	Aminoisobutyric acid
	Nle :	Norleucine
	β-Ala :	β-Alanine
	hPTH :	Human PTH
	Fmoc :	9-Fluorenylmethoxycarbonyl
35	Nva :	Norvaline
	Abu :	α-Aminobutyryl acid

By the amino acid substitution in the PTH(1-34) as described above, the resistance to various proteases is increased and the persistence of the activity in blood is obtained. This is achieved by, for example, substituting Aib for the 1st-position of PTH(1-34), and D-α-amino acid for the 12th-position of PTH(1-34).

40 The position around the 12-position Gly is considered to have the β-turn structure. The substitution of a D-α-amino acid for the Gly, particularly of a bulky D-α-amino acid such as D-Leu, D-Trp or D-Val, contributes to the stabilization of this structure, and the peptide chain is prevented from being digested by a protease at this position. Substitution of naturally occurring hydrophobic amino acid for Met at 8th or 18th position of PTH(1-34) increases resistance to oxidation, and is useful in prevention of reduction or elimination of activity of the peptide. Further, the affinity of the PTH derivatives for receptors is increased and high PTH activity is expressed by the substitution of amino acid residues at other positions. For example, the 11th position of PTH(1-34) is originally Leu. However, it is more preferable that the amino acid having an aromatic chain such as Phe, is substituted for Leu. Substitution for the 25th, 26th and 27th position basic amino acid of PTH(1-34), especially substitution of Gln or Leu for the 27th Lys; and substitution of 2-(1,3-dithiolane-2-yl)-50 Trp for the 23rd Trp bring about high PTH activity expression. It is understood, that the typical examples of amino acid substitution are not intended to limit the scope of the invention.

#### Example 1 Synthesis and Purification of PTH (1-34) Active Fragment Analogues

55 The peptides were synthesized in accordance with a modified method of the solid phase peptide synthesis developed by R. B. Merrifield, R. B. Merrifield, *Adv. Enzymol.* 32, 221-296 (1969), and an automatic peptide synthesizer 430A (Applied Biosystems) was used. Protected peptide-resins were synthesized using protocols specified by Applied Biosystems. Protected amino acid-p-Oxymethyl-

phenylacetamidomethyl resins (polystyrene-1% divinylbenzene) are used as starting materials when analogues having free carboxylic acids as carboxyl termini are desired, and 4-methylbenzhydryl resins are used as starting materials when analogues of carboxylamides are desired, and protected amino acids were condensed thereto successively. In order to protect an  $\alpha$ -amino group of each amino acid on condensation, 5 a tertiary-butyloxycarbonyl (BOC) group was used. Side functional groups were protected in the following manner. Hydroxyl groups of serine and threonine were protected as O-benzyl ethers, a hydroxyl group of tyrosine as a p-bromobutyloxycarbonyl ester, carboxyl groups of glutamic acid and aspartic acid as benzyl esters, imidazole nitrogen of histidine with benzyloxymethyl, a side chain amino group of lysine with 2-chlorobutyloxycarbonyl, a guanidine functional group of arginine with a p-toluenesulfonyl group, and 10 indoleimine of tryptophan with a formyl group. All amino acids were obtained from Applied Biosystems Japan and Bachem Chemicals.

After all of the amino acids were condensed on the resin, the protected peptide resin was taken out of the synthesizer and dried. The peptide resin (1 g) was allowed to react with anhydrous hydrogen fluoride (8 ml) containing p-cresol (1 ml), 1,2-ethanedithiol (1 ml) and 2-mercaptopuridine (100 mg) at 0°C for 2 hours. 15 After completion of reaction, hydrogen fluoride was removed by distillation and the residue was washed with diethyl ether to remove most of additives. The peptide was extracted with 3% acetic acid (10 ml), and the resin was removed by filtration. The filtrate was purified by gel filtration using a Sephadex G-25 column. The conditions of gel filtration were as follows: column size: 2.8X60 cm; detecting wavelength: 230 or 280 nm; solvent: 3% acetic acid; flow rate: 40 ml/hour. Fractions containing the peptide were collected and then 20 lyophilized. The resulting powder sample was further purified by reversed phase high performance liquid chromatography [column: YMC-pack, A-324 ODS (10X250 mm); eluting solvent A: 0.1% trifluoroacetic acid-99.9% water; eluting solvent B: 0.1% trifluoroacetic acid-99.9% acetonitrile; linear gradient elution program: 0 minute (90% A + 10% B), 30 minutes (60% A + 40% B) (if necessary another elution program may be used); elution rate: 1.6 ml/minute; detecting wavelength: 230 or 280 nm]. Peak fractions containing the 25 desired pure product were collected, and passed through a Bio RAD AGIX8 column (acetate form, 1.8 x 5 cm). The eluate was combined with the washings, and acetonitrile was removed therefrom by distillation, followed by lyophilization. The peptides thus obtained, the result of amino acids analysis and the retention times on HPLC are shown in Table 1.

In Table 1, a and b are as follows:

30 a: The peptides were hydrolyzed in a tube sealed with 6 N hydrochloric acid under reduced pressure, in the presence of 4% thioglycolic acid at 110°C for 24 hours, and then subjected to amino acid analysis. Theoretical values are designated in parentheses.

b: Names of compounds (no NH<sub>2</sub> at the terminus means COOH):

- (1) (Leu<sup>18</sup>)hPTH(1-34)
- (2) (Aib<sup>1</sup>)hPTH(1-34)
- (3) (Phe<sup>11</sup>)hPTH(1-34)
- (4) (D-Trp<sup>12</sup>)hPTH(1-34)
- (5) (Leu<sup>8</sup>)hPTH(1-34)NH<sub>2</sub>
- (6) (D-Tyr<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>
- (7) (D-Ser<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>
- (8) (D-Leu<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>
- (9) (3-(2-naphthyl)-D-Ala<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>
- (10) (Ser<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>
- (11) (Phe<sup>11</sup>,Leu<sup>18</sup>)hPTH(1-34)NH<sub>2</sub>
- (12) (Leu<sup>8</sup>,Phe<sup>11</sup>,Leu<sup>18</sup>)hPTH(1-34)NH<sub>2</sub>
- (13) (Lys<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>
- (14) (Phe<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>
- (15) (Arg<sup>19,21</sup>)hPTH(1-34)NH<sub>2</sub>
- (16) (3-(2-naphthyl)-Ala<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>
- (17) (His<sup>26</sup>)hPTH(1-34)NH<sub>2</sub>
- (18) (His<sup>25</sup>)hPTH(1-34)
- (19) (Gln<sup>27</sup>)hPTH(1-34)
- (20) (Arg<sup>18,21</sup>,2-(1,3-dithiolan-2-yl)-Trp<sup>23</sup>)hPTH(1-34)NH<sub>2</sub>
- (21) (Leu<sup>27</sup>)hPTH(1-34)
- (22) (Lys<sup>11</sup>)hPTH(1-34)

c: Retention time of the peptides by high performance liquid chromatography. Analysis conditions: VISTA 5000 high performance liquid chromatography (Varian) linked to 712W autosampler (Waters) was used. Column: YMC-303 ODS (4.6x250mm); Eluent: A, 0.1% trifluoroacetic acid-99.9% water; B, 0.1%

trifluoroacetic acid-99.9% acetonitrile; Eluent concentration gradient program: 0 minute(80% A + 20% B),  
30 minutes(50% A + 50% B); Flow rate 0.7 ml/minute; detective wave length 280 nm.

Table 1-1 Amino acid composition of PTH(1-34)analogues(a)

5

	<u>amino acid</u>	<u>peptide (b)</u>					
		(1)	(2)	(3)	(4)	(5)	(6)
10	A s x	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
15	S e r	2.44(3)	1.57(2)	2.23(3)	2.45(3)	2.36(3)	2.48(3)
20	G l x	5.28(5)	5.30(5)	4.99(5)	5.22(5)	5.24(5)	5.30(5)
25	G l y	1.03(1)	1.02(1)	1.04(1)		1.02(1)	
30	V a l	2.37(3)	2.77(3)	2.75(3)	2.87(3)	2.61(3)	2.79(3)
35	M e t	0.98(1)	1.91(2)	1.91(2)	1.91(2)	0.93(1)	1.83(2)
40	I l e	0.92(1)	0.92(1)	0.89(1)	1.00(1)	0.88(1)	0.95(1)
45	L e u	6.53(6)	5.03(5)	4.07(4)	5.07(5)	6.19(6)	5.10(5)
50	P h e	1.01(1)	1.01(1)	2.02(2)	1.05(1)	1.03(1)	1.02(1)
55	L y s	3.09(3)	3.04(3)	3.03(3)	2.94(3)	3.07(3)	3.05(3)
60	H i s	2.80(3)	2.88(3)	2.86(3)	2.80(3)	2.80(3)	2.81(3)
65	T r p	0.90(1)	1.09(1)	1.06(1)	1.90(2)	0.96(1)	0.92(1)
70	A r g	2.00(2)	1.97(2)	1.98(2)	1.99(2)	2.02(2)	1.96(2)
75	A i b		1.04(1)				1.02(1)
80	T y r						
85	H P L C retention time (minutes)	c 24.2	-	-	-	24.6	24.0

45

50

55

Table 1-2 Amino acid composition of PTH(1-34)analogues(a)

5

	amino acid	peptide (b)				
		(7)	(8)	(9)	(10)	(11)
10	A s x	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
	S e r	3.39(4)	2.35(2)	2.45(3)	3.29(4)	2.07(3)
15	G l x	5.17(5)	5.08(5)	5.31(5)	5.14(5)	4.80(5)
	G l y				1.03(1)	0.83(1)
20	V a l	2.83(3)	2.73(3)	2.58(3)	2.55(3)	2.43(3)
	M e t	1.90(2)	1.90(2)	2.11(2)	2.10(2)	1.03(1)
25	I l e	0.94(1)	0.85(1)	0.90(1)	0.91(1)	0.92(1)
	L e u	5.04(5)	5.97(6)	4.98(5)	3.92(4)	4.69(5)
30	P h e	1.05(1)	1.00(1)	1.07(1)	1.06(1)	1.70(2)
	L y s	2.98(3)	2.93(3)	2.81(3)	2.81(3)	2.57(3)
35	H i s	2.78(3)	2.81(3)	2.67(3)	2.66(3)	2.30(3)
	T r p	1.06(1)	0.86(1)	0.89(1)	0.70(1)	0.90(1)
40	A r g	2.01(2)	1.96(2)	1.88(2)	1.79(2)	1.61(2)
	A i b					
45	T y r					
	H P L C retention time					
50	(minutes) c	21.9	26.4	28.1	20.8	27.1
55						

Table 1-3 Amino acid composition of PTH(1-34)analogues(a)

	<u>amino acid</u>	<u>peptide (b)</u>					
		(12)	(13)	(14)	(15)	(16)	(17)
10	A s x	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
	S e r	2.07(3)	1.99(2)	2.51(3)	2.55(3)	2.55(3)	2.58(3)
	G l x	4.83(5)	4.71(5)	4.94(5)	3.95(4)	4.98(5)	5.05(5)
15	G l y	1.01(1)	0.96(1)	1.01(1)	1.02(1)	1.03(1)	1.04(1)
	V a l	2.65(3)	2.63(3)	2.73(3)	1.80(2)	2.73(3)	2.75(3)
	M e t		1.66(2)	2.12(2)	2.12(2)	1.90(2)	1.91(2)
20	I l e	0.81(1)	0.67(1)	0.84(1)	0.86(1)	0.86(1)	0.91(1)
	L e u	5.97(6)	3.92(4)	4.08(4)	5.08(5)	4.07(4)	5.08(5)
	P h e	1.99(2)	1.06(1)	2.04(2)	1.04(1)	1.03(1)	1.00(1)
25	L y s	2.92(3)	3.76(4)	2.96(3)	2.88(3)	3.03(3)	2.00(2)
	H i s	2.53(3)	2.45(3)	2.69(3)	2.68(3)	3.09(3)	4.07(4)
	T r p	0.65(1)	0.79(1)	0.87(1)	0.86(1)	0.92(1)	0.91(1)
30	A r g	1.93(2)	2.21(2)	1.94(2)	3.84(4)	1.96(2)	1.91(2)
	A i b						
	T y r						
H P L C ret time							
		28.4	20.8	28.1	26.6	24.8	23.4

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Table 1-4 Amino acid composition of PTH(1-34)analogues(a)

	<u>amino acid</u>	<u>peptide (b)</u>				
		(18)	(19)	(20)	(21)	(22)
10	A s x	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
	S e r	2.58(3)	2.72(3)	2.51(3)	2.99(3)	2.49(3)
	G l x	5.07(5)	6.27(6)	3.92(4)	4.90(5)	5.04(5)
	G l y	1.04(1)	1.00(1)	0.99(1)	1.31(1)	1.08(1)
	V a l	2.76(3)	2.76(3)	1.78(2)	2.77(3)	2.78(3)
	M e t	1.91(2)	1.91(2)	2.03(2)	1.82(2)	2.12(2)
	I l e	0.89(1)	0.90(1)	0.87(1)	0.91(1)	0.91(1)
	L e u	5.11(5)	5.05(5)	5.04(5)	5.90(6)	3.96(4)
	P h e	1.02(1)	0.96(1)	1.04(1)	1.00(1)	1.01(1)
	L y s	3.02(3)	1.91(2)	2.79(3)	1.87(2)	3.86(3)
25	H i s	3.64(4)	2.66(3)	2.64(3)	2.79(3)	2.74(3)
	T r p	0.95(1)	0.84(1)	0.84(1)	0.79(1)	0.85(1)
	A r g	0.98(1)	1.86(2)	3.83(4)	1.91(2)	1.90(2)
	A i b					
	T y r					
H P L C retention time						
40	(minutes) c	24.8	26.0	28.2	29.2	23.6

Example 2 Synthesis and purification of (Arg<sup>19,21</sup>,2-(1,3-dithiolane-2-yl)Trp<sup>23</sup>)hPTH(1-34)NH<sub>2</sub>

560 mg of peptide resin synthesizing (Arg<sup>19,21</sup>)hPTH(1-34)NH<sub>2</sub> was allowed to react with anhydrous hydrogen fluoride (5 ml) containing p-cresol (620 µl) and ethanedithiol(620 µl) at 0°C for 2 hours. After hydrogen fluoride was removed by distillation, the residue was washed with diethyl ether containing 0.1% 2-mercaptoethanol. The resulting product was dried and the peptide was extracted with trifluoroacetic acid (5 ml), and the resin was removed by filtration. Ether was added to the filtrate and the resulting precipitate was separated by filtration and washed with ether. 280 mg of the crude peptide was obtained. The peptide was purified by reverse phase high performance liquid chromatography. The conditions of the chromatography were as follows: Column, YMC-pack, A-324 ODS (10x250 mm); Eluent A, 0.1% trifluoroacetic acid - 99.9% water; Eluent B, 0.1% trifluoroacetic acid - 99.9% acetonitrile; Eluent concentration gradient program, 0 minute (70% A + 30% B), 40 minutes (55% A + 45% B); flow rate: 1.6 ml/minute. Two large peaks (retention times 17.0 minutes and 18.2 minutes) were observed in the chromatography. The former peak (retention time 17.0 minutes) was recovered and changed to acetate by an ion-exchange resin. The acetate was then lyophilized to obtain 4.9 mg of (Arg<sup>19,21</sup>)hPTH(1-34)NH<sub>2</sub>. After hydrolysis, the resulting product

shows the correct amino acid composition in the amino acid analysis. The ultraviolet absorption of the product shows a specific curve characteristic of a peptide comprising tryptophan.

6.9 mg of compound was obtained from the latter peak. Amino acid analysis of the compound after acid-hydrolysis showed the correct composition, but amino acid analysis after trypsin-amino peptidase M digestion showed only 0.28 residue of tryptophan and the glutamic acid was detected 0.65 residue less than the theoretical value. Ultraviolet absorption curve for the digested compound showed a peak of 289 nm and a valley of 255 nm. As a result, tryptophan side chain of the compound is deduced to be modified. The following process showed that 1,3-dithiolan linked to the C2 carbon of the side chain indole of tryptophan.

A compound (4mg) obtained from the peak at a retention time 18.2 minutes in the above high performance liquid chromatography was dissolved into 60mM sodium hydrogen carbonate pH8.0(2.6ml). TPCK-trypsin(160 µg) was added to the solution and reacted for 24 hours at 37°C, and then was inactivated by heating for 6 minutes at 100°C. Aminopeptidase-M (0.5mg) was added to the resulting solution adjusted to pH7 and incubated at 37°C for 24 hours and then the enzyme(0.5 mg) was further added thereto. After an additional 48 hour period, the buffer (10 ml) and the enzyme (1 mg) were added thereto and reacted for 70 hours. The resulting product was subjected to reverse phase high performance liquid chromatography to isolate a modified triptophan. Column, YMC D-ODS-5 S5 120A (20X250m); the same eluent; Eluent program, 0 minute (80%A + 20%B), 40 minutes (65%A + 35%B); Flow rate, 5ml/minute; detected at 280 nm. The isolated compound showed a maximum ultraviolet absorption at 289nm. Amino acid analysis after hydrolysis with 6N HCl containing 4% thioglycolic acid showed triptophan. When the product was subjected to high resolution FAB-mass spectrum (Nihon Denshi, Japan; AX-505W TYPE double convergence mass spectrometer), a peak at 309.0734(M + H<sup>+</sup>) as observed and a molecular formula C<sub>14</sub>H<sub>17</sub>N<sub>2</sub>O<sub>2</sub> S<sub>2</sub> was deduced. Further about 30 ng of the compound was subjected to <sup>1</sup>H-MNR(Nihon Denshin, JNM-GX400).

(DMSO-d<sub>6</sub>),  $\alpha$ -CH  $\delta$  = 4.06 (1H, dd like),  $\beta$ -CH<sub>2</sub> 3.54(1H, dd), 3.30(1H, dd); 1-NH 10.88 (1H); 5-CH 7.50 (1H, d); 6-CH 7.30 (1H, t like); 7-CH 7.20 (1H, t like); 8-CH 7.68 (1H, d); dithiolan 2CH 6.14 (1H, S); dithiolan 4CH<sub>2</sub> and 5CH<sub>2</sub> 3.64 (2H, m) and 3.49 (2H, m).

The above data show that the isolated hPTH(1-34)NH<sub>2</sub> analogue has 2-(1,3-dithiolan-2-yl)-tryptophan at 23th position.

### Example 3 Assay of Biological Activity of PTH (1-34) Analogues

The biological activity of the peptide analogues was evaluated by a modified version of the method reported by Shigeno et al. in The Journal of Biological Chemistry 263, 18369-18377 (1988). A culture solution (Hank's solution, containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin and 0.5 mM isobutylmethyl-xanthine) containing 0.01, 0.1, 1, 10 or 100 nM analogue was added in an amount of 100 µl to a mouse cranial bone-derived osteoblast-like cell strain, MC3T3-E1 cells, cultivated on a 96-well multiplate (Nunclon, Nunc), followed by reaction at room temperature for 30 minutes. After addition of 100 µl of 0.2 N hydrochloric acid, the mixture was immersed in boiling water for 2.5 minutes, and cyclic adenosine monophosphate (cAMP) produced by a PTH receptor was extracted from the cells. The total cAMP in the culture solution and the cells was assayed using a commercial radioimmunoassay kit (cyclic AMP [<sup>125</sup>I] kit "Du Pont-Daiichi", Daiichi Kagaku Yakuhin). An increase in cAMP production depending on the concentration of the human PTH (1-34) added as a standard was observed in each case. The biological activity of the PTH (1-34) peptide analogines is shown in Table 2.

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Table 2

Biological Activity of PTH(1-34) Partial Peptides (Represented by Relative Activity to hPTH(1-34))	
5	hPTH(1-34) 1.00
	[Leu <sup>18</sup> ]hPTH(1-34) 0.4
	[Aib <sup>1</sup> ]hPTH(1-34) 1.7
	[Phe <sup>11</sup> ]hPTH(1-34) 1.1
	[Leu <sup>8</sup> ]hPTH(1-34)NH <sub>2</sub> 0.5
10	[D-Ser <sup>12</sup> ]hPTH(1-34)NH <sub>2</sub> 0.8
	[D-Leu <sup>12</sup> ]hPTH(1-34)NH <sub>2</sub> 0.5
	[3-(2-naphthyl)-D-Ala <sup>12</sup> ]hPTH(1-34)NH <sub>2</sub> 0.9
	[Ser <sup>11</sup> ]hPTH(1-34)NH <sub>2</sub> 0.8
	[Leu <sup>8</sup> , Phe <sup>11</sup> , Leu <sup>18</sup> ]hPTH(1-34)NH <sub>2</sub> 0.9
15	[Lys <sup>11</sup> ]hPTH(1-34)NH <sub>2</sub> 1.1
	[Phe <sup>11</sup> ]hPTH(1-34)NH <sub>2</sub> 1.3
	[Arg <sup>19,21</sup> ]hPTH(1-34)NH <sub>2</sub> 0.9
	[3-(2-naphthyl)-Ala <sup>11</sup> ]hPTH(1-34)NH <sub>2</sub> 1.7
	[His <sup>26</sup> ]hPTH(1-34)NH <sub>2</sub> 0.9
20	[His <sup>25</sup> ]hPTH(1-34) 1.0
	[Gln <sup>27</sup> ]hPTH(1-34) 2.5
	[Arg <sup>19,21</sup> ,2-(1,3-ditiolan-2-yl)-Trp <sup>23</sup> ]hPTH(1-34)NH <sub>2</sub> 1.7
	[Leu <sup>27</sup> ]hPTH(1-34) 1.2

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## Sequence Listing

SEQ ID NO:1

SEQUENCE LENGTH: 34

SEQUENCE TYPE: amino acid

TOPOLOGY: Linear

MOLECULE TYPE: Peptide

FEATURE: Partial Peptide

SEQUENCE DESCRIPTION:

Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn

1 5 10 15

Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

20

25

30

Asn Phe

SEQ ID NO:2

SEQUENCE LENGTH: 34

SEQUENCE TYPE: amino acid

TOPOLOGY: Linear

MOLECULE TYPE: Peptide

FEATURE:

LOCATION:1

OTHER INFORMATION:Xaa= Ser or Aib,

LOCATION:8

OTHER INFORMATION:Xaa= Met or naturally occurring hydrophobic amino acid

LOCATION:11

OTHER INFORMATION:Xaa= Leu, Ser, Lys or aromatic amino acid

LOCATION:12

OTHER INFORMATION:Xaa= Gly or D-amino acid

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LOCATION:13

OTHER INFORMATION:Xaa= Lys or Leu

5

LOCATION:18

OTHER INFORMATION:Xaa= Met or naturally occurring hydrophobic amino acid

10

LOCATION:19

OTHER INFORMATION:Xaa= Glu or basic amino acid

LOCATION:21

15

OTHER INFORMATION:Xaa= Val or basic amino acid

LOCATION:23

20

OTHER INFORMATION:Xaa= Trp or 2-(1,3-ditionlan-2-yl) Trp

LOCATION:25

OTHER INFORMATION:Xaa= Arg or His

25

LOCATION:26

OTHER INFORMATION:Xaa= Lys or His

LOCATION:27

30

OTHER INFORMATION:Xaa= Lys , Gln or Leu

LOCATION:34

35

OTHER INFORMATION:Xaa= Phe or Phe-NH,

SEQUENCE DESCRIPTION:

Xaa Val Ser Glu Ile Gln Leu Xaa His Asn Xaa Xaa Xaa His Leu Asn

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1

5

10

15

Ser Xaa Xaa Arg Xaa Glu Xaa Leu Xaa Xaa Xaa Leu Gln Asp Val

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His Asn Xaa

34

50 Claims

1. A peptide or salts thereof represented by the formula

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$R_1$ -Val-Ser-Glu-Ile-Gln-Leu- $R_2$ -His-Asn- $R_3$ - $R_4$ - $R_5$ -  
 His-Leu-Asn-Ser- $R_6$ - $R_7$ -Arg- $R_8$ -Glu- $R_9$ -Leu- $R_{10}$ - $R_{11}$ - $R_{12}$ -  
 Leu-Gln-Asp-Val-His-Asn- $R_{13}$

wherein R<sub>1</sub> represents Ser or Aib; R<sub>2</sub> represents Met or a naturally occurring hydrophobic amino acid; R<sub>3</sub> represents Leu, Ser, Lys or an aromatic amino acid; R<sub>4</sub> represents Gly or a D- $\alpha$ -amino acid; R<sub>5</sub> represents Lys or Leu; R<sub>6</sub> represents Met or a naturally occurring hydrophobic amino acid; R<sub>7</sub> represents Glu or a basic amino acid; R<sub>8</sub> represents Val or a basic amino acid; R<sub>9</sub> represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R<sub>10</sub> represents Arg or His; R<sub>11</sub> represents Lys or His; R<sub>12</sub> represents Lys, Gln or Leu; and R<sub>13</sub> represents Phe or Phe-NH<sub>2</sub>; except that simultaneously R<sub>1</sub> consists of Ser, R<sub>2</sub> consists of Met, R<sub>3</sub> consists of Leu, R<sub>4</sub> consists of Gly, D-Ala or D-Pro, R<sub>5</sub> consists of Lys, R<sub>6</sub> consists of Met, R<sub>7</sub> consists of Glu, R<sub>8</sub> consists of Val, R<sub>9</sub> consists of Trp, R<sub>10</sub> consists of Arg, R<sub>11</sub> consists of Lys and R<sub>12</sub> consists of Lys.

- <sup>0</sup> R<sub>7</sub> consists of Cys.  
R<sub>12</sub> consists of Lys.

  2. The peptide of claim 1 wherein a naturally occurring hydrophobic amino acid in R<sub>2</sub> and R<sub>6</sub> is Leu, Ile, Val, Phe or Trp.
  3. The peptide of claim 1 wherein an aromatic amino acid in R<sub>3</sub> is Phe,  $\beta$ -naphthyl Ala, Trp or Tyr.
  4. The peptide of claim 1 wherein a D- $\alpha$ -amino acid is D-Leu, D-Ile, D-Nle, D-Val, D-Ser, D-Ser(But), D-Abu, D-Thr, D-Nva, D-Met,  $\beta$ -naphthyl-D-Ala, D-Trp, D-Tyr, D-Lys, D-Lys(Fmoc), D-Phe or D-Asn.
  5. The peptide of claim 1 wherein a D- $\alpha$ -amino acid is a neutral amino acid.
  6. The peptide of claim 5 wherein neutral D- $\alpha$ -amino acid is D-Ser, D-Leu, D-naphthyl Ala, D-Trp, D-Asn or D-Tyr.
  7. The peptide of claim 1 wherein basic amino acid in R<sub>7</sub> and R<sub>8</sub> is Arg, Lys, Asn or His.
  8. The peptide of claim 1 wherein the peptide of the formula [1] simultaneously has 10 to 12 amino acids optionally selected from a group consisting of Ser for R<sub>1</sub>, Met for R<sub>2</sub>, Leu for R<sub>3</sub>, Gly for R<sub>4</sub>, Lys for R<sub>5</sub>, Met for R<sub>6</sub>, Glu for R<sub>7</sub>, Val for R<sub>8</sub>, Trp for R<sub>9</sub>, Arg for R<sub>10</sub>, Lys for R<sub>11</sub>, Lys for R<sub>12</sub> and Phe for R<sub>13</sub>.
  9. The peptide of claim 1 which is (Leu<sup>18</sup>)hPTH(1-34), (Aib<sup>1</sup>)hPTH(1-34), (Phe<sup>11</sup>)hPTH(1-34), (D-Trp<sup>12</sup>)-hPTH(1-34), (Leu<sup>8</sup>)hPTH(1-34)NH<sub>2</sub>, (D-Tyr<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>, (D-Ser<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>, (D-Leu<sup>12</sup>)hPTH(1-34)NH<sub>2</sub>, (3-(2-naphthyl-D-Ala<sup>12</sup>))hPTH(1-34)NH<sub>2</sub>, (Ser<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>, (Phe<sup>11</sup>,Leu<sup>18</sup>)hPTH(1-34)NH<sub>2</sub>, (Leu<sup>8</sup>,Phe<sup>11</sup>,Leu<sup>18</sup>)hPTH(1-34)NH<sub>2</sub>, (Lys<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>, (Phe<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>, (Arg<sup>19,21</sup>)hPTH(1-34)-NH<sub>2</sub>, (3-(2-naphthyl)-Ala<sup>11</sup>)hPTH(1-34)NH<sub>2</sub>, (His<sup>25</sup>)hPTH(1-34), (Gln<sup>27</sup>)hPTH(1-34), (Arg<sup>19,21</sup>,2-(1,3-dithiolane-2-yl)Trp<sup>23</sup>)-(His<sup>26</sup>)hPTH(1-34)NH<sub>2</sub>, (His<sup>25</sup>)hPTH(1-34) or (Lys<sup>11</sup>)hPTH(1-34).



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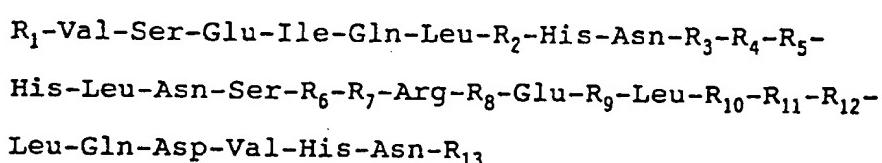
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D-50462 Köln (DE)

(54) Parathyroid hormone derivatives.

(55) Disclosed are peptides and salts thereof represented by general formul



wherein R<sub>1</sub> represents Ser or Aib; R<sub>2</sub> represents Met or a naturally occurring hydrophobic amino acid; R<sub>3</sub> represents Leu, Ser, Lys or an aromatic amino acid; R<sub>4</sub> represents Gly or a D- $\alpha$ -amino acid; R<sub>5</sub> represents Lys or Leu; R<sub>6</sub> represents Met or a naturally occurring hydrophobic amino acid; R<sub>7</sub> represents Glu or a basic amino acid; R<sub>8</sub> represents Val or basic amino acid; R<sub>9</sub> represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R<sub>10</sub> represents Arg or His; R<sub>11</sub> represents Lys or His; R<sub>12</sub> represents Lys, Gln or Leu; and R<sub>13</sub> represents Phe or Phe-NH<sub>2</sub>; except that simultaneously R<sub>1</sub> consists of Ser, R<sub>2</sub> consists of Met, R<sub>3</sub> consists of Leu, R<sub>4</sub> consists of Gly, D-Ala or D-Pro, R<sub>5</sub> consists of Lys, R<sub>6</sub> consists of Met, R<sub>7</sub> consists of Glu, R<sub>8</sub> consists of Val, R<sub>9</sub> consists of Trp, R<sub>10</sub> consists of Arg, R<sub>11</sub> consists of Lys and R<sub>12</sub> consists of Lys.

The parathyroid hormone (1-34) analogues are useful in hormone therapy.

EP 0 477 885 A3



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 91116303.8						
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.s)						
A	<u>EP - A - 0 341 963</u> (MERCK & CO. INC) * Claims 1-4 * --	1-9	C 07 K 13/00 C 07 K 15/06 A 61 K 37/30						
A	<u>EP - A - 0 293 159</u> (MERCK & CO. INC) * Claims 1-3 * --	1-9							
A	<u>EP - A - 0 293 158</u> (MERCK & CO. INC) * Claims 1-4 * --	1-9							
A	<u>DE - A - 3 428 942</u> (TOYO JOZO K.K.) * Claim 1 * -----	1-9							
			TECHNICAL FIELDS SEARCHED (Int. Cl.s)						
			C 07 K 7/00 C 07 K 13/00 C 07 K 15/00 C 07 C 103/00 C 12 N 5/00 C 12 N 15/00 A 61 K 37/00						
<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>VIENNA</td> <td>08-07-1993</td> <td>SCHARF</td> </tr> </table> <p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone  Y : particularly relevant if combined with another document of the same category  A : technological background  O : non-written disclosure  P : intermediate document</p> <p>T : theory or principle underlying the invention  E : earlier patent document, but published on, or after the filing date  D : document cited in the application  L : document cited for other reasons  &amp; : member of the same patent family, corresponding document</p>				Place of search	Date of completion of the search	Examiner	VIENNA	08-07-1993	SCHARF
Place of search	Date of completion of the search	Examiner							
VIENNA	08-07-1993	SCHARF							